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14. ABSTRACT

During the first year of current funding period, we have focused our study on the identification of BRCA1 regulated genes in human breast cancer cells. By combining the genome-wide microarray study and gene-specific approaches, we have discovered a group of genes that were significantly repressed or stimulated by BRCA1, and several genes in this group, such as TIMP-1, S100P, and GABBR1 have been implicated in the development of breast cancer. We have also found that transcriptional regulation by BRCA1 required gene-specific involvement of its cofactor, COBRA1. These findings will serve as the basis for us to explore the role of BRCA1 in transcriptional regulation in the presence of DNA damage. It also provides targets for investigating the dynamic association of BRCA1, the transcriptional machinery, and DNA damage checkpoint protein complex with chromatin templates under the stress conditions.

15. SUBJECT TERMS
BRCA1, tumor suppression, transcriptional regulation, DNA damage response

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Table of Contents

INTRODUCTION

Breast cancer 1 (*BRCA1*) is a tumor suppressor gene for hereditary breast and ovarian cancers (1). The BRCA1 protein interacts with numerous protein partners and is implicated in multiple cellular functions, among which DNA damage responses and transcriptional regulation are the two most extensively studied activities of BRCA1 (2). While functional studies of BRCA1 in these two directions are historically carried out independently, both activities of BRCA1 are likely to take place in the same cellular context. However, it is not clear whether these two BRCA1-mediated chromosomal processes are directly connected both spatially and temporarily. And furthermore, it remains unknown as to the potential mechanisms used by BRCA1 to coordinate these two nuclear events.

To address this broad question, we have proposed in the original application to focus our study on the functional connection between transcription-coupled repair (TCR) and transcriptional regulation, two events that have been reported to be BRCA1-regulated (3,4) and could conceivably take place on the same chromosomal template when BRCA1-regulated genes are insulted by DNA damage. In particular, we were interested in examining whether BRCA1 would facilitate TCR at its transcriptionally regulated gene targets. If BRCA1 indeed preferentially promote TCR at its regulated gene loci, then what will be the mechanism for such a function of BRCA1? In light of the mounting publications that document BRCA1's interaction with various components of the transcription apparatus as well as the DNA damage repair machinery, we hypothesized that BRCA1 might play an active role in orchestrating and ensuring a smooth transition between these two processes before and after DNA damage on its regulated gene loci. To test this hypothesis, we have proposed experiments first to determine the role of BRCA1 in TCR and transcription in response to DNA damage (Task 1). In addition, we will perform chromatin-immunoprecipitation (ChIP) assay to directly explore the impact of BRCA1 on the dynamic association of transcription and repair protein complex at these gene loci (Task 2).

Obviously, the finding of BRCA1's involvement in TCR was the key theoretical basis for our proposal. In addition, the TCR-specific repair assay would serve as a powerful tool for our proposed work. However, the recent retraction of two key papers from the original lab that reported the BRCA1-TCR link has cast serious doubts on the validity of the assay as well as the claim that BRCA1 plays a direct role in TCR (5-8). These unfortunate events, which occurred beyond our control, have not only weakened the rationale for pursuing the purported role of BRCA1 in TCR, but also significantly dampened the likelihood of success in using the TCR repair assay. After careful review of the circumstance and consultation with Dr. Rong Li, my thesis advisor, I strongly feel that a potential role of BRCA1 in TCR should not be pursued as a viable paradigm for studying the functional connection between BRCA1-dependent DNA damage responses and transcriptional regulation. Hence, the TCR-related experimental design is now removed from the future work. (Also see Dr. Rong Li's supporting letter in appendices.)

While the retracted work has caused significant confusion and distraction to the BRCA1 field, the function of BRCA1 in DNA damage response in general is based on a wealth of unequivocal evidence from numerous laboratories over the past decade. Therefore, a potential role of BRCA1 in coordinating damage response and transcription remains to be an important mechanistic question that is worth investigating. Rather than using the TCR assay as a way of studying BRCA1 function following DNA damage, we will focus on the impact of BRCA1 on the dynamics of transcription factors and checkpoint proteins at multiple BRCA1-associated genomic loci (see the revised statement of work in appendices). To achieve this overall objective, we will first conduct gene expression profiling study to examine the impact of BRCA1 on transcription in the absence and presence of genotoxic insult to the genome in human breast cancer cell lines (Task 1). As proposed in the original application, interesting phenotypic traits identified from this high-throughput experiment will be rigorously verified and further characterized by gene-specific approaches, such as quantitative RT-PCR, nuclear run-on assay, and mRNA stability studies, to determine the step at which BRCA1 may influence gene expression before and after DNA damage. On an independent but related front, we will combine the chromatin-immunoprecipitation assay and the tiling array technique ("ChIP-on-chip") to investigate in a genome-wide manner how BRCA1, the transcriptional machinery, and the DNA damage checkpoint complexes are recruited to these BRCA1-regulated gene loci before and after DNA damage (Task 2). Control and BRCA1 knockdown cells will also be included in experiments to determine whether and how BRCA1 physically affects the machinery switch processes at its target genes during DNA damage responses. Integration and comparison of results from both tasks will thus provide a comprehensive view to the potential connection between both functions of BRCA1. As described in more details below, we have made quite solid progress in the first task. By performing a microarray study on a breast cancer cell line in the absence of DNA damage, we have identified a group of genes that are significantly up- or down-regulated by BRCA1. After being verified by quantitative RT-PCR analysis, these genes will serve as the foundation for carrying out the above-mentioned studies.

BODY

I. Identification of genes that are regulated by BRCA1 in breast cancer cells

To explore the impact of BRCA1 on gene expression in the presence of DNA damage, we have first conducted a microarray study to determine the expression profile of BRCA1-reduced T47D breast cancer cells. To obtain the BRCA1 knockdown cells, we have used the retrovirus-dependent shRNA expression system from Oligoengine to stably introduce BRCA 1-specific shRNA into the T47D cells through puromycine selection. As a control, virus carrying shRNA for EGFP was used for parallel infection. Cofactor of BRCA1 (COBRA1) is a newly identified binding partner of BRCA1 from our laboratory (9). Recently, we have found that COBRA1 repressed estrogen-dependent transcription of TFF-1 (10). The same gene has also been reported to be inhibited by BRCA1 (11). In light of this functional similarity, we have decided to include COBRA1 in our analysis as well to examine a potential joint action of BRCA1 and COBRA1 in transcriptional regulation. Therefore, we infected this pair of cell lines with shCOBRA-

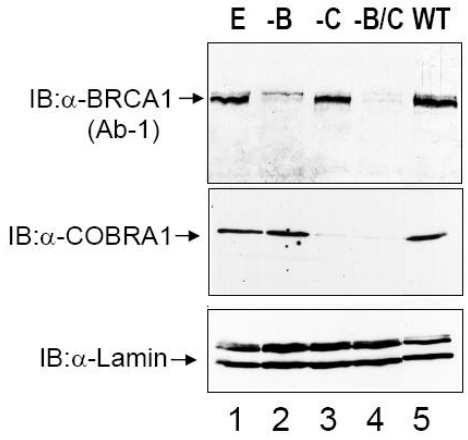

Figure 1. **Immunoblot analysis showing the decreased expression of BRCA1 and COBRA1 protein in the shRNA reduced T47D cells lines**. Whole cell extracts were prepared and analyzed by Western blotting using antibodies against BRCA1(top panel), COBRA1(middle panel), lamin (bottom panel). Lamin was used as an internal control for protein loading. E: shEGFP control cells; -B: shBRCA1; -C: shCOBRA1; -B/C: shBRCA1 and shCOBRA1 double knockdown; WT: non-infected T47D cells.

expressing virus and selected with neomycine for COBRA1 single and BRCA1/COBRA1 double-knockdown pools. As revealed by Western Blot analysis shown in Figure 1, the protein levels of BRCA1 and COBRA1 were significantly reduced by their corresponding shRNAs. In addition, the expression level of BRCA1 was independent of COBRA1 reduction, and vice versa. We also included the non-infected T47D cells as an additional control to show the unaffected expression of BRCA1 and COBRA1 in the shEGFP control cells.

To determine the expression profile of the engineered cells, we cultured the shEGFP control, shBRCA1, shCOBRA1, and shBRCA1/shCOBRA1 pools to 70% confluence. Total RNA was then isolated from the duplicated samples and subjected to microarray analysis using the human gene array chips (HU-133A) from Affymetrix. When compared with the shEGFP cells, a total of 287 genes were modulated by at least two-fold with p-values of 0.05 or less in the shBRCA1, shCOBRA1, and shBRCA1/shCOBRA1 cells. As shown in Figure 2, a heat map measuring the similarity in gene expression profile of the four cell lines was generated for the 287 genes via the Pearson correlation analysis. A careful examination of the heat map revealed three pairs of expression patterns of the 287 genes. (1) For genes in group A and group D, reduction of BRCA1 or COBRA1 alone resulted in change of the mRNA level in the same direction (decrease for genes in group A and increase for group D), and knockdown of both proteins led to a further decrease (group A) or increase (group D) of the mRNA level, or maintained the similar level of reduction or increase. Therefore, based on the similar effect of BRCA1 and COBRA1 on gene expression, it is reasonable to believe that BRCA1 and COBRA1 collaboratively regulate the genes from these two groups. (2)

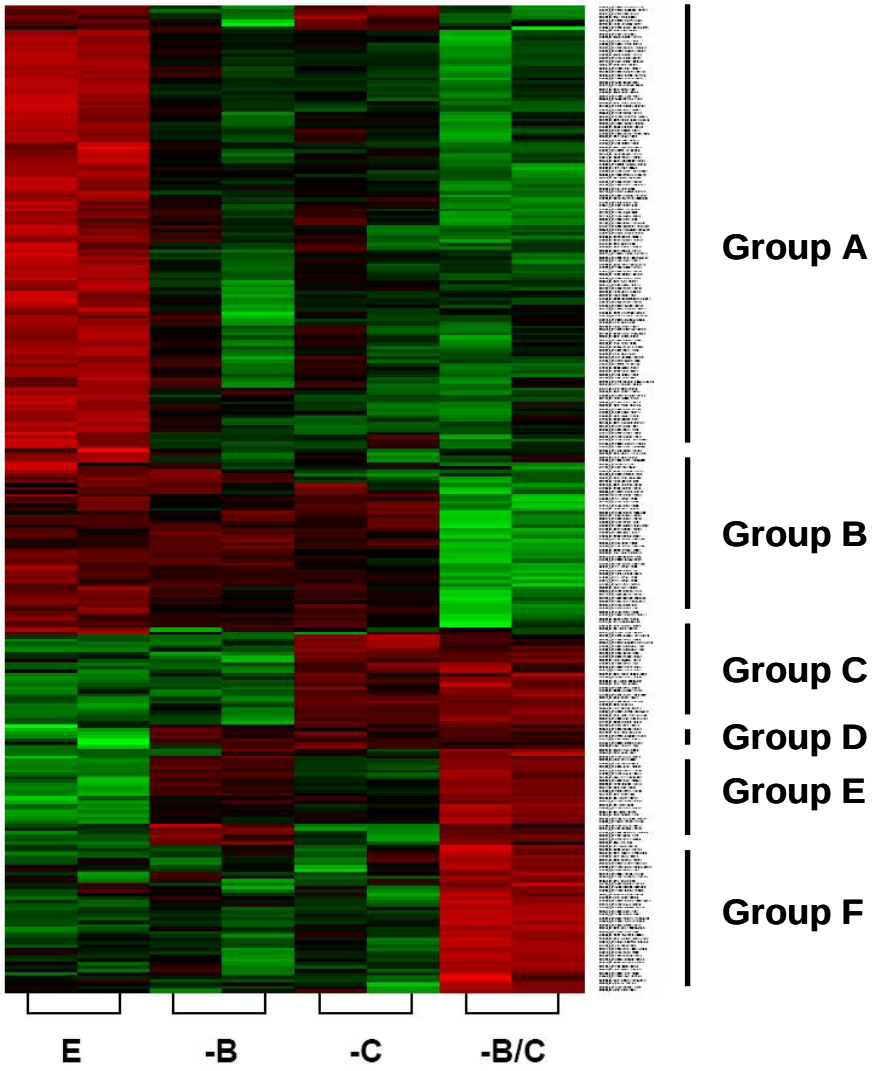

Figure 2. **Heat map of BRCA1 and COBRA1 regulated genes.** The classification is based on 278 filtered microarray genes using an algorithm based on the Pearson's correlation coefficient. Red color indicates a high level of expression whereas green indicates a low level of expression. Black color indicates no change in intensity. E: shEGFP control cells; -B: shBRCA1; -C: shCOBRA1; -B/C: shBRCA1 and shCOBRA1 double knockdown; WT: non-infected T47D cells.

For genes in group B and group F, reduction of either BRCA1 or COBRA1 alone was not sufficient to significantly change the expression. Only when both proteins were reduced could dramatic change be observed. Thus, although BRCA1 and COBRA1 could regulate gene expression in the same direction, their functions on these gene loci might be redundant, and therefore reduction of neither one would be sufficient for observing the apparent phenotype. (3) For genes in group C and group E, their expression was affected by the reduction of only one protein (group C was only affected by COBRA1 knockdown

and group E by BRCA1 reduction). Moreover, depletion of BRCA1 had no impact on COBRA1 regulated gene expression (compare E vs –C and –B vs –B/C), and so did knockdown of COBRA1. Hence, the regulations by BRCA1 and COBRA1 on these two sets of genes were independent with each other.

II. Validation of the microarray study with gene-specific quantitative RT-PCR analysis

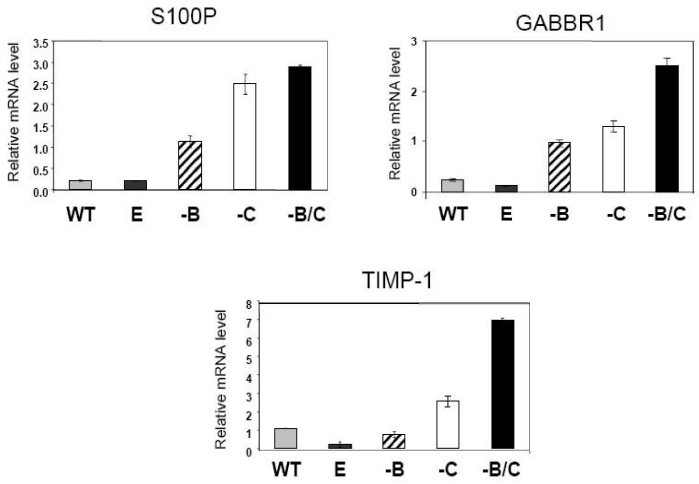

Figure 3. **BRCA1 and COBRA1 function to modulate transcription of specific endogenous target genes.** Total RNA was isolated for quantitative RT-PCR analysis with primer pairs for the individual genes. WT: non-infected T47D cells, E: shEGFP control cells; -B: shBRCA1; -C: shCOBRA1; -B/C: shBRCA1 and shCOBRA1 double knockdown.

Following the microarray study, we performed quantitative RT-PCR (qRT-PCR) analysis to further validate the effect of BRCA1 and COBRA1. The genes that were chosen for further verification include those that have been previously implicated in breast cancer, such as S100P (12), TIMP-1 (13), and GABBR1(14). We first confirmed the changes in gene expression by using the same RNA prepared for the microarray analysis (data not shown). We then re-generated the four shRNA-expressing knockdown cell pools and verified the knockdown effect again by RT-PCR. As shown in Figure 3, the expression level of all three genes was indeed elevated when BRCA1 or COBRA1 was reduced. Interestingly, for some genes such as TIMP1 and GABBR1, simultaneous knockdown of COBRA1 and BRCA1 resulted in a much greater effect on gene expression than knockdown of either gene alone (compare column "–B" and "–C" with "–B/C" for TIMP1), suggesting the involvement of both COBRA1 and BRCA1 in gene regulation at these loci. On the other hand, this additive effect of co-depletion of BRCA1 and COBRA1 was not apparent for the expression of S100P. In fact, the stimulatory effect of BRCA1 knockdown on S100P expression (compare E vs –B) was significantly reduced when COBRA1 was depleted from the cells (compare –C vs –B/C). Therefore, COBRA1 was required for BRCA1-dependent transcriptional regulation at this particular gene locus.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of BRCA1 regulated genes by microarray study in breast cancer cells
- Characterization of COBRA1's role in BRCA1-mediated gene expression
- Validation of BRCA1 regulated gene expression by qRT-PCR

REPORTABLE OUTCOMES

None

CONCLUSION

The preliminary findings obtained during the first year of the current funding period laid out a solid foundation for the continuing study of BRCA1's impact on transcriptional regulation in the presence of DNA damage. Identification of BRCA1 regulated genes also provides transcriptional targets for further investigating the dynamic association of BRCA1, transcription apparatus, and DNA damage checkpoint protein complexes before and after DNA damage. In the second year of the contract, we will extend the above-reported microarray study to explore the impact of BRCA1 on gene expression when the cells are challenged with genotoxic insults. In addition, we will perform the ChIP assay to unravel the association pattern of BRCA1 and its binding partners under the native and stressed conditions.

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